Boesenbergia rotunda extract accelerates human keratinocyte proliferation through activating ERK1/2 and PI3K/Akt kinases

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ABSTRACT

Boesenbergia rotunda (BR) has long been used as tradition medicine. For its pharmacological effects on wound healing, previous studies in an animal model provided convincing results that the ethanolic extract from the rhizome of this plant can stimulate wound healing. However, the mechanism about how this plant promotes wound healing at the molecular level has not been elucidated. As a step towards the development of wound healing agents, our current study utilized a human keratinocyte cell line (HaCaT) as an *in vitro* model to define the potential molecular mechanisms of BR extract in enhancing wound-healing. Our HPLC results showed that BR extract contained kaempferol as one of its potential compounds. The extract strongly promoted wound healing of HaCaT cell monolayer. This effect was eventually defined to be regulated through the ability of BR extract to induce cell proliferation. At the signaling level, we discovered that BR extract rapidly activated ERK1/2 and Akt phosphorylation upon the addition of the extract. Additionally, our experiments where specific inhibitors of MEK (U0126) and PI3K (LY294002) were utilized verified that BR enhanced cell proliferation and wound healing through stimulating the MAPK and PI3K/Akt signal transduction pathways. Moreover, direct inhibition of keratinocyte DNA synthesis by mitomycin C (MMC) could completely block the proliferative effects of BR extract. Nevertheless, data from Transwell migration assay revealed that BR extract did not promote keratinocyte migration. Altogether, we provided more evidence that BR possesses its wound healing-promoting action through the activation of proliferation and survival pathways, and our study suggests that BR is an interesting candidate to be developed as a wound healing-promoting agent.

1. Introduction

A wound is described as “any disruption of the integrity of skin, mucous membrane, or organ tissue” [1], and a process called “epithelialization is required for a success of wound healing [2]. Keratinocytes are responsible cells of the epidermis which regulate the restoration process of the epidermis after injury, and this process is defined as “re-epithelialization” [2]. In particular, the epidermis is a stratified epithelium composed of several layers of keratinocytes that can ascend from proliferative state in the basal layer to differentiated state during migration through the granular layer, and finally become dehydrated and flattened dead cell corneocytes in the cornified layer [3,4]. These differentiated processes are involved with major mitogen-activated protein (MAP) kinase pathways activated by various stimuli, including calcium influx, epidermal growth factor (EGF), and tumor necrosis factor (TNF) [5]. Furthermore, the mitogen-activated protein kinase kinase kinase 1 (MEKK1) conveys wound signals to activate several different genes associated with extracellular matrix (ECM) homeostasis, epithelial cell migration, and wound re-epithelialization [6]. Similarly, phosphatidylinositol-3-kinase (PI3K) is generally involved in growth factor-mediated signal transduction that controls cell proliferation, transformation, protein trafficking, actin cytoskeletal organization, and apoptosis by activation of different effectors [7,8]. One important effector of PI3K is the serine/threonine kinase Akt which plays an important role in the maintenance of a viable and proliferative epidermis with preservation of the skin architecture [9]. Moreover, the modulation of keratinocyte migration is regulated by multiple factors such as growth factors, cytokines, integrins, keratins, matrix metalloproteinases, chemokines, and extracellular macromolecules [10,11]. The balance between these growth factors and cytokines controls all progressive phases of wound

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healing events, however, if non-healing wounds fail to progress, they tend to remain in a prolonged inflammatory phase. Therefore, chronic wounds resulted from imbalances of wound proteases and their inhibitors may lead to degradation of growth factors and cytokines in the damage area and consequently delays the healing process [2,12]. Since the exact mechanisms of action of keratinocyte migration and proliferation remain not fully elucidated, reliable and effective drugs that could accelerate the aforementioned process have yet to be developed. Interestingly, despite the development of standard medicines, many plants with healing properties have been used as traditional and complementary medicines. Recently, a range of plant extracts and other products used as traditional wound healing agents have been investigated [13].

Boesenbergia rotunda (BR) or fingerroot or Krachai in Thai, a well- known traditional herb, is a ginger species that belongs to Zingiberaceae family [14]. The pharmacological and biological effects of BR include antimicrobial activities [15–17], inhibition of biofilm formation [18,19], antioxidant activities [20,21], antiulcer effects [20], obesity treatment [22], anti-cancer activities [23,24], antiviral activities [25], anti-inflammatory activities [26,27], and inhibition of platelet-activating factor receptor binding effects [28]. *Boesenbergia rotunda* contains active flavonoids and chalcone derivatives [29]. Kaempferol is one of the major compounds in this plant which has been reported that topical application of kaempferol accelerated the healing rate of the incisional and excisional wounds in diabetic and nondiabetic rats [30]. Since kaempferol is abundantly present in *Boesenbergia rotunda*, it is reasonable to discover that this plant has wound-healing activity. This statement is confirmed by previous studies reporting that ethanolic extracts of BR rhizomes significantly accelerated wound healing enclosure in rats, and the healed wound contained less inflammatory cells and higher level of collagen with angiogenesis compared to a control group [31]. This study strongly suggested that BR has potential wound-healing promoting functions. However, its exact molecular mechanisms of action, especially for its specific effects on human keratinocytes, remain largely unexplored.

The present study aimed to evaluate whether the extract of BR has effects on inducing cell proliferation, cell migration, and wound healing activity using human keratinocyte, HaCaT cell line, as a study model. Specifically, our study investigated the effects of BR at molecular level by examining vital markers in the MAPK signal transduction pathway, including activation of ERK1/2, P38, and JNK as well as Akt which is a key player in the PI3K/Akt signal transduction pathway. Moreover, cell behavioral changes such as cell proliferation and cell migration after BR treatment were evaluated. Since BR extract exhibited the ability to accelerate wound healing in animals in a previous study, and our current study provided convincing results in human keratinocyte, we strongly believe that BR extract could be a promising candidate as a great resource for future production of a potential wound healing agent.

2. Materials and methods

2.1. Plant material and extraction of Boesenbergia rotunda rhizomes

Fresh rhizomes of *Boesenbergia rotunda* (BR), were collected from the area of Chiang Mai province, Thailand in July 2019. The plant was authenticated by the CMU staff botanist and the voucher specimen (number 009724) was deposited at the Herbarium of the Faculty of Pharmacy, Chiang Mai University. After washing, the plants were cut into small pieces, dried in a temperature-controlled oven at 55 °C and ground into fine powder, which then was macerated with 95 % ethanol for 24 h at room temperature (RT). After filtration (Whatman No.1) (Sigma-Aldrich, USA) the solvent was evaporated in a rotary evaporator at 40 °C to remove ethanol. The BR extract stock solution was freshly prepared using DMSO (Sigma-Aldrich, USA) prior to each assay. A stock BR extract solution of 1 g/mL was prepared by dissolving 1 g of the extract in 1 mL of 100 % DMSO. For each experiment the stock was pre- diluted in media prior to each treatment. *2.2. High performance liquid chromatography (HPLC)*

Potential active compound profile of BR extract was investigated by HPLC using a C18 column (250 mm × 4.6 mm, 5 µm) (Sigma-Aldrich, USA). Gradient elution was performed using two solvents; A (1% acetic acid in water) and B (100 % acetonitrile) for the detection. Twenty micro liters of the 500 µg of BR extract was dissolved in 1 mL of methanol and injected into the column with a flow rate 0.7 mL/min and detected at 280 nm. The content of the active compound in extract was determined by comparison of peak area and retention time with standard curves of various concentrations of kaempferol standard (Tokyo Chemical Industry, Japan).

2.3. Cell culture

An immortalized human epidermal keratinocyte cell line, HaCaT cell, was purchased from Cell Lines Service GmbH (Eppelheim, Germany). The cells were cultured in complete media, which was Dulbecco's modified Eagle's media (DMEM) (Thermo Fisher Scientific, USA), supplemented with 10 % fetal bovine serum (Merck KGaA, Germany) and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin) (Thermo Fisher Scientific, USA), and incubated under a humidified atmosphere of 37 °C, 5% CO_2.

2.4. Cell viability assay

Effects of the *Boesenbergia rotunda* extract on viability of HaCaT cells was performed by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to obtain the range of toxic and nontoxic concentrations, HaCaT cells were seeded in 96-well plates at a density of 2 × 10^5 cells per well for 24 h in complete media. HaCaT cells were treated with BR extract at various concentrations (0.2–250 µg/mL) or with vehicle (DMSO at 0.001–

0.05 %) for 48 h in complete media or serum-free media. Then, cell viability was performed by MTT assay for assessing cell metabolic activity. Briefly, 25 μL of MTT reagent (5 mg/ mL) was added into each conditioned well containing 200 μL of culture media for 1− 2 h. Then, the culture supernatants were discarded, and 100 μL of 100 % DMSO was added to solubilize the formazan complex. The plate was then measured for the absorbance at 570 nm using a microplate reader (BioTek Instruments, USA). Three different non- cytotoxic concentrations (3.75, 7.5, and 15 μg/mL) were selected for all experiments. *2.5. Cell proliferation assay*

Colony forming assay was performed to visualize cell proliferation using a phase contrast microscope. HaCaT cells were seeded in 24-well plates in complete DMEM and incubated for 24 h at 37 °C, 5% CO_2. Cells were treated with BR at 3.75, 7.5, and 15 μg/mL in serum-free media for 48 h. The pictures of BR-treated HaCaT cells at different time points (0, 24, and 48 h) were photographed by Axio Vert.A1 microscope (Carl Zeiss, Germany), with 10× magnification, and micrographs were captured with the Zen 2.6 (blue edition) Software for the Zeiss Axiocam 506 color microscope camera. Moreover, cell counting technique was used to verify the changes of cell number. Specifically, HaCaT cells were seeded in 24-well plates in complete DMEM and incubated for 24 h at 37 °C, 5% CO_2. Cells was treated with BR extract in serum-free DMEM at the selected non-toxic concentrations (3.75, 7.5, and 15 μg/mL). The total number of cells at different time points (0, 24, and 48 h) and different concentration was counted by using a haemacytometer.

2.6. Cellular wound healing activity assay

HaCaT cells were seeded and cultured in 24-well plates for 24 h, serum-starved, and then the mechanical scratch wound was created by using a 200-mL sterile pipette tip. Cells were washed once with sterile PBS and treated with different concentrations of BR extract. Images of the cellular wound healing were photographed at different time points (0, 3, 6, 18, and 24 h). The complete closure of scratched wounds was considered to be the completion of the migration process. The migrated areas were determined and analyzed using the ImageJ software. In addition, this experiment was performed with the presence of mitomycin C (Sigma-Aldrich, USA), U0126 (MEK-1,2 inhibitor) (Cell Signaling Technology, USA), or LY294002 (PI3K inhibitor) (Cell Signaling Technology, USA) in order to examine the possible molecular mechanism action of BR extract for enhancing cell growth and survival. *2.7. Transwell migration assay*

A cell culture insert was used to confirm the effect of BR extract on cell migration. HaCaT cells were seeded in the upper chamber and cultured in complete DMEM for 24 h. On the next day, cells in the upper chamber were treated with different concentrations of BR extract in serum-free DMEM (SFM), and the upper chambers was put into the (lower) wells containing DMEM with 5% FBS and incubated for 24 h. Cells were fixed with absolute methanol for 5 min at RT and stained with 0.5 % crystal violet in methanol for 30 min. The upper chamber was washed for 3 times with water, and cells attached to the surface inside the chamber were removed with a cotton swab. The stained cells attached at the other site of the chamber were captured with a light microscope and analyzed with the ImageJ software. Additionally, the nuclei of the cells were stained with DAPI to visualize and confirm the cell distribution under a fluorescent microscope.

2.8. Western blot analysis

To examine the effects of BR on MAPK and PI3K/Akt signaling pathways, HaCaT cells were treated with BR at different concentrations for 15 min or treated with 15 μg/mL at various time points (0–24 h). To verify the specific signal transduction pathways by which BR extract may stimulate, we also performed the experiments with the presence of specific inhibitors for PI3K (LY294002) (catalog number 9901)(Cell Signaling Technology, Boston, MA, USA) and for MEK-1,2 (U0126) (catalog number 9903)(Cell Signaling Technology, Boston, MA, USA). After treatment, cells were lysed by adding 1× reducing Laemmli buffer. The cell lysates were collected, heated at 95 °C for 5 min, separated by SDS-PAGE, and transferred onto polyvinylidene difluoride (PVDF) membranes (GE Healthcare Life Science, USA). Membranes were blocked with 5% skim milk in Tris-buffered saline with Tween 20 (TBS-T) (0.02 M Tris− HCl, pH 7.6, 0.0137 M NaCl, and 0.05 % Tween 20) (all reagents from Sigma-Aldrich, USA) at RT for 1 h. Membranes were then incubated with an appropriate primary antibody (Cell Signaling Technology, USA) diluted in 5% BSA (Merck KGaA, Germany) in TBS-T at 4 °C overnight. Primary antibodies consist of a dilution of a phosphospecific rabbit anti-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody (catalog number 4370), a mouse anti-p44/42 MAPK (Erk1/2) (3A7) antibody (catalog number 9107), a phosphospecific mouse anti-p38 MAPK (Thr180/Tyr182) antibody (catalog number 9216), a rabbit anti-p38 MAPK (D13E1) antibody (catalog number 8690), a phosphospecific rabbit anti-SAPK/JNK (Thr183/Tyr185) antibody (catalog number 4668), a mouse anti-SAPK/JNK antibody (catalog number 9252), a phosphospecific rabbit anti-Akt (Ser473) antibody (catalog number 4060), a mouse anti-Akt (40D4) antibody (catalog number 2920) or a mouse anti-β-actin antibody (catalog number MA1115) (Boster Biological Technology, Pleasanton, CA, USA). After washing with TBS-T, membranes were incubated with secondary antibodies (Li− COR Biosciences, Lincoln, NE, USA); an anti-mouse IgG conjugated with IRDye®800CW (catalog number 926–32210) (1:10000) or an anti-rabbit IgG conjugated with IRDye®680RT (catalog number 926–68071) (1:10000) at RT for 2 h. The positive protein bands were detected using an Odyssey® CLx Imaging System (LI− COR Biosciences, USA). The immunoreactive bands were quantified using the ImageJ software.

2.9. Immunofluorescence study

Immunofluorescence was performed to visualize Akt or ERK phosphorylation and Ki-67 expression in the nuclei of HaCaT cells. For detecting the phosphorylation status of Akt and ERK1/2, HaCaT cells were grown on glass cover slips for 24 h and then treated with BR extract for 15 min. For determining the expression of Ki-67, the cells on coverslips were treated with BR extract for 24 h. After treatment, cells were fixed with 4% paraformaldehyde (Sigma-Aldrich, USA) dissolved in PBS for 15 min at RT. Cells were then washed thrice with PBS for 5 min, each time and permeabilized with 0.3 % Triton X-100 in PBS for 5 min. The cells were then blocked with 1% bovine serum BSA/PBS for 1 h. Cells were incubated with appropriate primary antibodies (Cell Signaling Technology, USA) overnight at 4 °C. Primary antibodies included a phosphospecific rabbit anti-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody (catalog number 4370), a rabbit anti-Ki-67 (D3B5) antibody (catalog number 9129) and a phosphospecific rabbit anti-Akt (Ser473) antibody (catalog number 4060). After washing three times with PBS, cells were incubated with appropriate secondary antibodies (Alexa488-conjugated anti-rabbit IgG or Alexa594-conjugated goat anti-rabbit IgG) (Thermo Fisher Scientific, USA) for 2 h, in the dark at RT. Cells were counterstained with 1 μg/mL of DAPI (Sigma-Aldrich, USA) for nuclear staining for 1 h. Then cells were washed three times with PBS and one time with distilled water. Finally, sample cover slips were mounted with Fluoromount-G (SouthernBiotech, USA) and the sample coverslips were observed under a fluorescent microscope, Axio Vert.A1 (Carl Zeiss, Germany), with 100× magnification. Micrographs were captured with the Zen 2.6 (blue edition) Software for the Zeiss Axiocam 506 color microscope camera.

2.10. Statistical analysis

All experimental data were represented as mean ± SD and analyzed by one-way analysis of variance (ANOVA) with Tukey's post hoc multiple comparisons on RAW data reads using SPSS software (SPSS Inc., Chicago, IL, USA). A p-value ($p < 0.05$) was considered as statistically significant.

3. Results

3.1. HPLC chemical profile of kaempferol in Boesenbergia rotunda extract

Since it is known that BR extract contains a flavonoid, kaempferol, as one of the active compounds [29], and this flavonoid has been shown to have strong wound healing effects in diabetic and nondiabetic excisional wounds in rat models [30], we thus determined the existence and the quantity of kaempferol in BR extract. The results demonstrated that HPLC fingerprint chromatogram of the extract showed the major peak of kaempferol at the retention time (RT) of 33.450 (Fig. 1A). This retention time detected in the extract was compatible with that of standard kaempferol (RT = 33.401) (Fig. 1B). Furthermore, when BR extract and kaempferol standard was combined, the perfect single peak of kaempferol was clearly seen (RT = 33.286) (Fig. 1C). For qunatitative analysis, we compared the amount of kaempferol with the standard curve and found that the amount of the compound in BR extract was 208.04 ± 9.86 mg/g extract. This number was then calculated to be approximately 21 % w/w of kaempferol found in BR extract.

3.2. Effects of Boesenbergia rotunda extract on cell viability of HaCaT cells

We initiated our study by performing MTT cell viability assay to specifically select the concentrations that are not toxic to the cells for all further experiments. We performed the experiments in BR extract- treated cells cultured in complete media and serum-free media to differentiate the response behaviors of the cells in different culture conditions. Results showed that BR extract at 15.6 μg/mL (and lower) did not affect cell viability of HaCaT cells cultured in complete media or serum-free media. However, the extract with concentrations higher than 31.3 μg/mL significantly reduced the viability of HaCaT cells (Fig. 2A and B). Interestingly, we found that BR extract at the range of non-toxic concentrations showed an increased trend of metabolic activity in HaCaT cells cultured in serum-free media but not in complete media. Specifiacally, BR extract at 3.9, 7.8, and 15.6 μg/mL significantly increased cell viability of HaCaT cells cultured in serum-free media to 20.94 ± 12.18 %, 29.54 ± 10.63 %, and 49.39 ± 13.37 %, respectively (Fig. 2B). Cells treated with DMSO (as a vehicle control) at consistent concentrations existing in the BR extract-treated group did not cause any difference in cell viability (Fig. 2A and B). These results reflect the possibility that BR extract may increase cell viability via its ability to

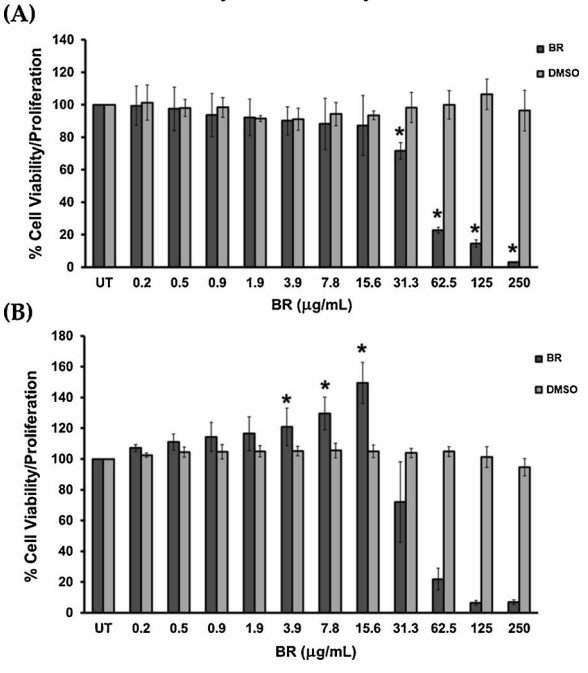


Fig. 2. Cell viability test by MTT assay in HaCaT cells treated with various concentration of BR extract for 48 h. (**A**) Cell viability assay for HaCaT cells treated with BR extract in complete media; (**B**) Cell viability assay for HaCaT cells treated with BR extract in serum-free media. DMSO was used as a vehicle control. Data are representatives of three individual replicates; * $p < 0.05$ (in comparison to the untreated group, UT).

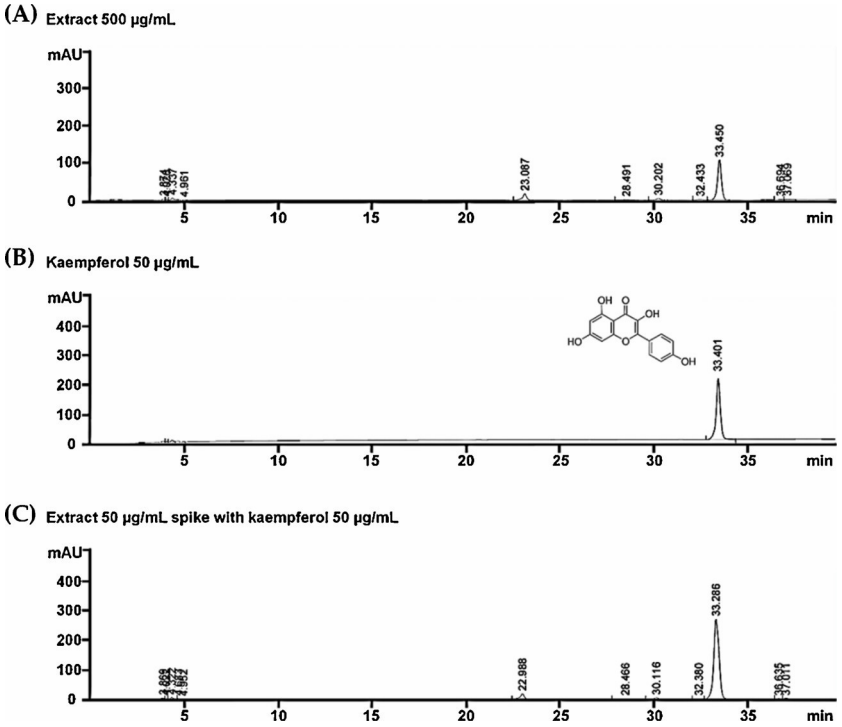


Fig. 1. High performance liquid chromatography (HPLC) for compound analysis. (A) HPLC chromatogram of *Boesenbergia rotunda* (BR) ethanolic extract (500 µg/ mL). (B) HPLC chromatogram of kaempferol standard with its structure (50 µg/mL); C) HPLC chromatogram of the mixture of BR ethanolic extract (50 µg/mL) and kaempferol standard (50 µg/mL); the retention time at 33 min indicates the existence of kaempferol.

stimulate the proliferation of HaCaT cells.

3.3. BR extract accelerates scratch wound closure in HaCaT cell layers

As a step towards the evaluation of the wound healing effects of BR extract on human keratinocyte cell, we performed scratch wound healing assay in HaCaT cell treated with BR extract over the course of 24 h. Data demonstrated that BR extract at 15 μg/mL drametically prompted significant HaCaT cell migration at the time points after 6 h of incubation compared to those of DMSO-treated group (Fig. 3A and B). These results suggest the possibility that BR extract may accelerate wounded monolayer cultures of human keratinocyte by activating the process of cell proliferation or cell migration.

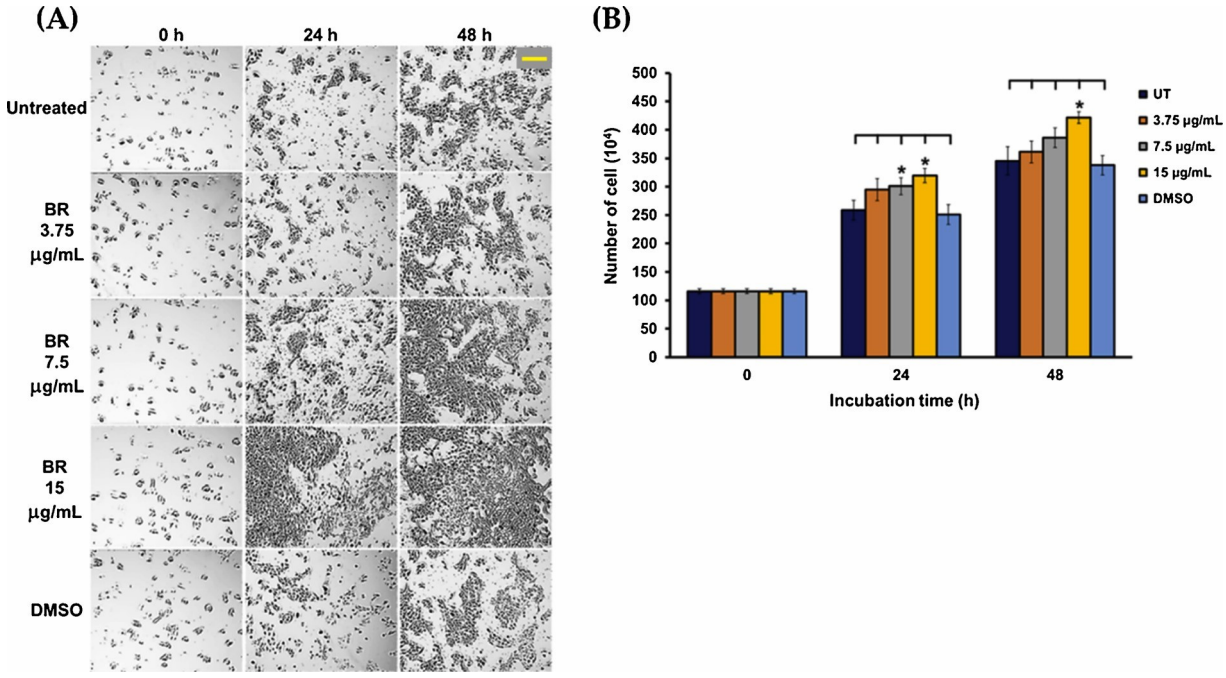
3.4. BR extract induces HaCaT cell proliferation

From our observation that BR extract could increase cell viability and wound healing of HaCaT cell monolayer, we hypothesized that these increased cell viability and scratch wound healing rate may be caused from an increase in HaCaT cell proliferation. Therefore, we designed an experiment to determine the ability of BR extract in inducing cell proliferation in the condition without serum by observing the proliferative behavior of HaCaT at different time points (0− 48 h) under a phase-contrast microscope. Additionally, cell counting was performed to quantify the difference in the number of cells in all groups. Results from these experiments revealed that at the 0 time point the distribution and the number of the cells were seen to be similar in control and experimental groups (Fig. 4A and B). As expected, at 24 h and 48 h it was found that the occupied surface areas on the culture plates and the number of HaCaT cells significantly increased in cells treated with BR extract at 7.5 and 15 μg/mL (Fig. 4A and B). To confirm our findings, we performed immunofluorescence study to examine the expression level and localization of Ki-67 protein in HaCaT cells. Ki-67 protein is associated with cell cycle progression and highly expressed in cells that are in the proliferative phase. Our immunofluorescence results clearly showed that HaCaT cells treated with BR extract exhibited high expression in their nuclei (Fig. 5a-c) in comparison to the basal expression of this protein seen in the nuclei of the DMSO-treated cells (Fig. 5d-f). These results indicate that BR extract has proliferation-enhancing effects on HaCaT cells.

3.5. BR extract rapidly activates MAPK and PI3K/Akt signal transduction pathways

We attemped to explore the possible molecular mechanisms of action of BR extract in inducing cell proliferation. Since it is well known that growth and survival signal transduction pathways are involved in cell proliferation and survival, we determined whether BR extract activates MAPK and PI3K/Akt signaling activation. Western blot analysis detecting the phosphorylation status of different kinases were performed for the lysates of HaCaT cells after incubation with 15 μg/mL of BR extract at various time points. As expected, BR extract could rapidly induce ERK1/2 phosphorylation after 2 min of incubation, and the activation reached the maximal level at 15 min after BR extract addition (Fig. 6A and B). Interestingly, after 30 min ERK1/2 phosphorylation was drastically reduced, and the second wave of ERK1/2 activation was observed between 1 h–24 h (Fig. 6A and B). Addtionally, we noticed similar activation pattern in Akt phosphirylation status, but the second Akt phosphorylation wave trended to exhibit a slightly stronger activation than the initial wave (Fig. 6A and C). The maximum fold change of kinase phosphorylation for ERK1/2 and Akt was approximately 2 and 3 folds, respectively (Fig. 6B and C). However, we discovered that BR extract did not induce phosphorylation of JNK and P38 kinases (Fig. 6A).

Moreover, we attempted to verify whether BR extract can stimulate ERK1/2 and Akt activation in a dose-dependent manner. We performed Western blot analysis similar to that of the time-dependent experiments by varying the concentration of BR extract from 3.75 to 15 μg/mL. Results from this experiment clearly demonstrated that phosphorylation of ERK1/2 and Akt was significantly increased when the concentration of BR extract was increased, and the total protein of these two kinases was not changed (Fig. 7A). We quantified the intensity of the immunoreactive bands of each kinase and found that the maximal activation of



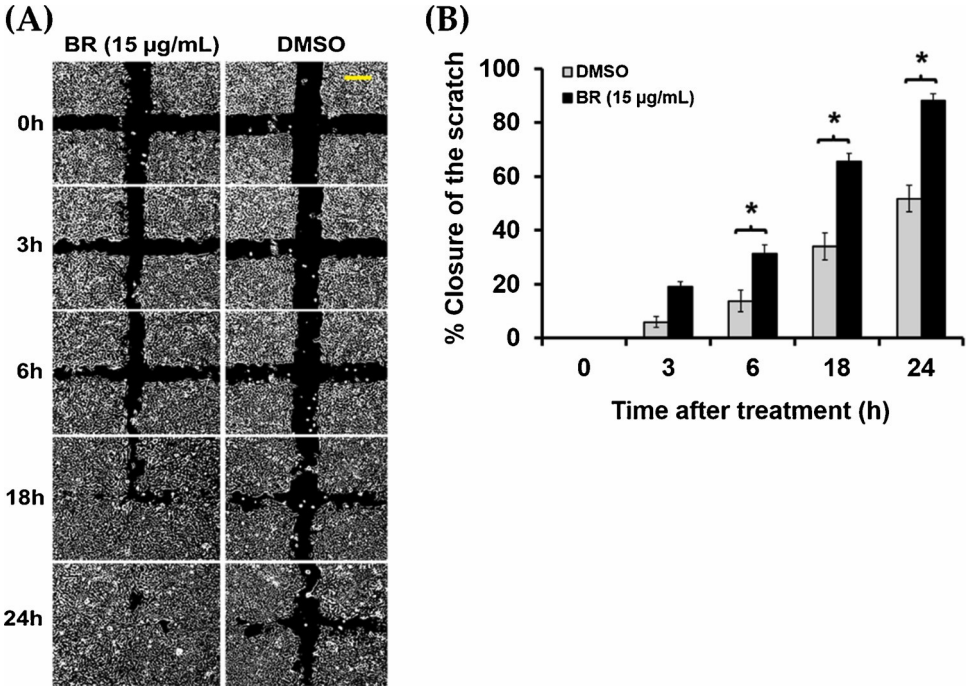


Fig. 3. Effect of BR extract on scratch wound closure in HaCaT cell monolayers. (**A**) Micrographs from a phase contrast microscope (10× magnification, scale bar =200 µm) capturing cell monolayer healing rate of HaCaT at 0, 3, 6, 18, and 24 h after treatment with 15 µg/mL of BR extract; (**B**) Quantification of the area occupied by HaCaT cells after various incubation times. Data are representatives of three replicates. * $p < 0.05$ (in comparison to DMSO control group).

Fig. 4. Effects of BR extract on HaCaT cell proliferation. (**A**) Colony forming assay of HaCaT cells treated with BR extract at different concentrations (3.75, 7.5, and 15 µg/mL) observed by a phase-contrast microscope (10× magnification, scale bar =200 µm) at different time points (0, 24, and 48 h); (**B**) Cell counting assay for HaCaT cells treated with BR extract at different concentrations and at different time points. Data represent 3 individual experiment. * $p < 0.05$ (in comparison to the untreated group).

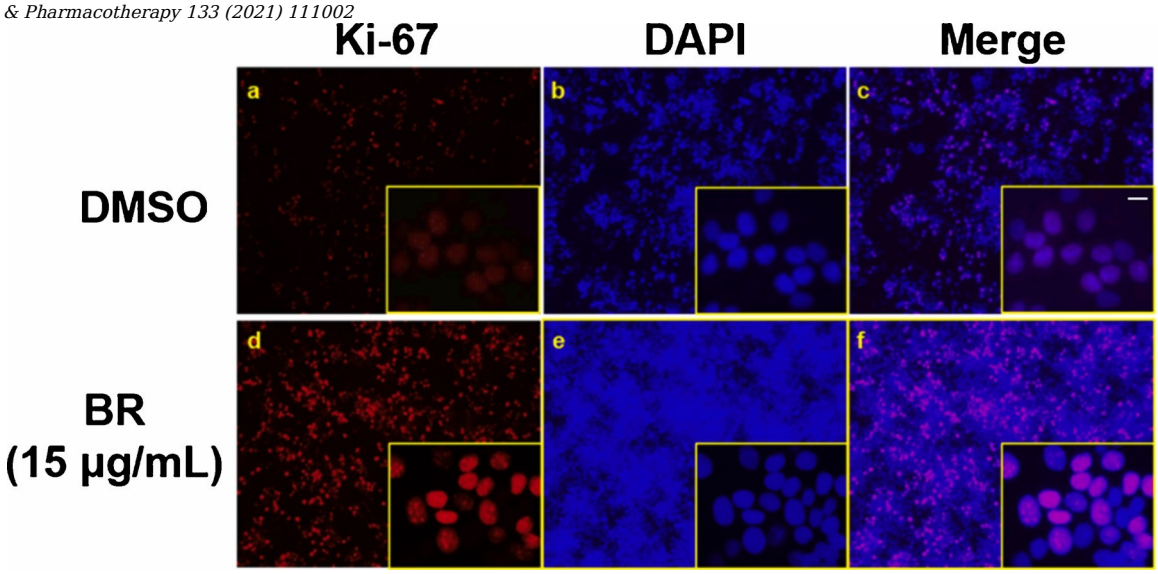


Fig. 5. Immunofluorescence study for effects of BR extract on Ki-67 (red) expression. HaCaT cells were counterstained with DAPI for nuclear staining (blue). Immunoflourescent images were captured at 40× magnification, and magnified views are shown on the right (100× magnification, scale bar =150 μm). Data are representatives of three replicates. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

ERK1/2 and Akt had a very similar pattern in which approximately 1.4–1.5 fold change after 15 min of treatment with BR extract was observed (Fig. 7B and C). These data strongly indicate that BR extract truly activates the growth and survival signal transduction pathways.

Additionally, analysis of pERK and pAkt activation in each cell upon BR extract treatment further verified that BR extract dramatically increased intracellular phosphorylation of these two kinases (Fig. 8A and B).

3.6. Inhibition of growth and survival signaling pathways suppresses proliferation-enhancing effects of BR extract

Since we observed that BR extract could intensely induce ERK1/2 and Akt phosphorylation, we believed that these two signaling pathways are mainly involved in promoting HaCaT cell proliferation. To ensure our hypothesis, we therefore designed experiments by using specific inhibitors of the PI3K/Akt and MAPK pathways. Data from our Western blot analysis confirmed that when LY294002 which is a specific inhibitor of PI3K was applied, complete inhibition of Akt but not ERK1/2 was observed in comparison to those of the cells treated with BR extract only

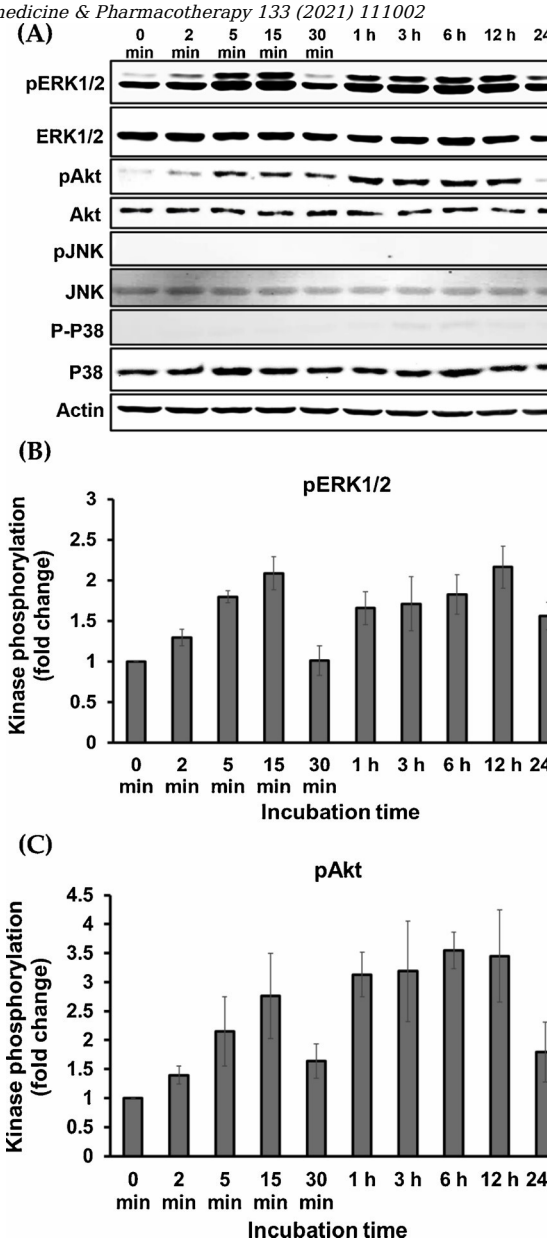


Fig. 6. Effects of BR extract on the activation of growth and survival signal transduction pathways. (**A**) Western blot analysis detecting the phosphorylation status of MAP kinases and Akt in HaCaT cells after treatment with 15 μg/mL of BR extract at different time points (0-24 h); Quantitative analysis of ERK1/2 phosphorylation (**B**) and Akt phosphorylation (**C**) upon BR extract treatment at different time points. The unphosphorylated form of each kinase was detected and used for normalization. Actin was used as an internal control. Data are the representative of 3 individual replicates.

(Fig. 9A and B). Similarly, when U0126 which is a specific MEK inhibitor was utilized, the phosphorylation of ERK1/2 but not Akt completely disappeared (Fig. 9C). Clearly, when we combined both LY294002 and U0126, the consequence of ERK1/2 and Akt phosphorylation was not seen (Fig. 9D). In order to be certain that the complete inhibition of these two pathways can lead to the suppression of BR extract's ability to promote cell proliferation, we performed a functional test (scratch wound assay) with the presence of LY294002 and U0126. As expected, data revealed that in comparison to the BR extract-treated group, U0126 alone could efficiently inhibit healing of the cell monolayer over the course of 24 h (Fig. 10A and B). Similar observation was witnessed in the case of BR extract-treated HaCaT cells with the presence of LY294002 (Fig. 10A and C). Undoubtedly, the combination of U0126 and

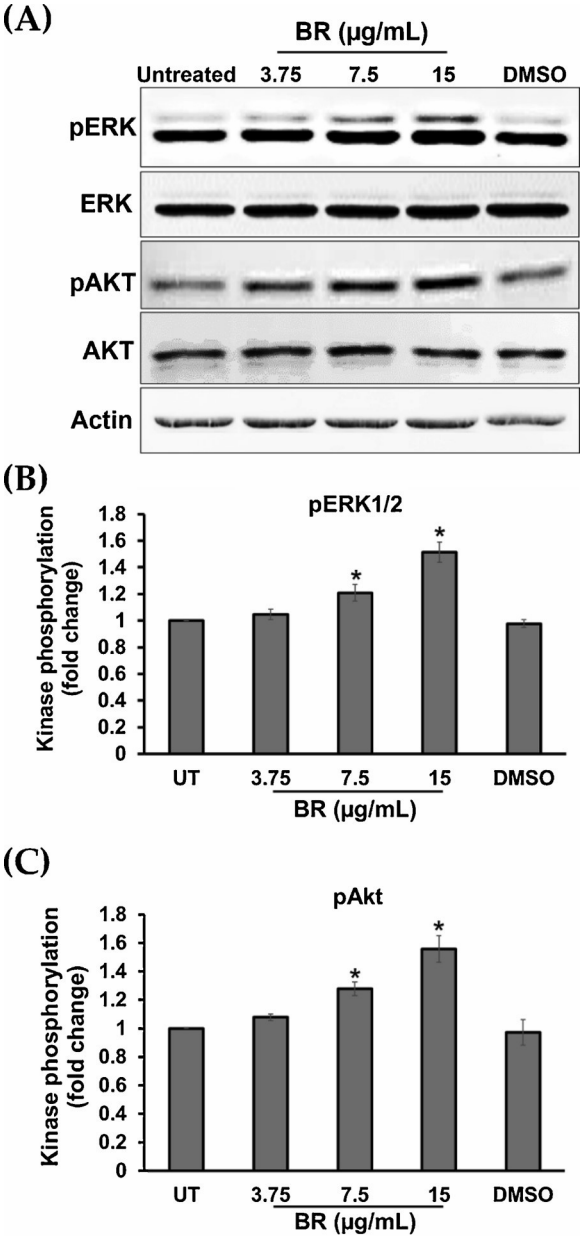


Fig. 7. Concentration-dependent effects of BR extract on ERK1/2 and Akt activation. (**A**) Western blot analysis for ERK1/2 and Akt phosphorylation in HaCaT cells treated with various concentrations of BR extract (3.75, 7.5, and 15 μg/mL) after 15 min of incubation. Quantitative analysis of ERK1/2 phosphorylation (**B**) and Akt phosphorylation (**C**). Data represent 3 independent experiments with * $p < 0.05$ (in comparison to the untreated group, UT).

LY294002 almost completely suppressed the closure of the scratch wound (Fig. 10A and D). To verify that direct inhibition of BR extract- induced cell proliferation can also result in inhibition of scratch wound healing, we used mitomycin C (MMC) which

is a well- characterized chemotherapeutic drug that potently inhibits DNA synthesis. Interestingly, MMC alone could completely abolish the ability of BR extract in enhancing cell proliferation as observed by a complete inhibition of monolayer wound closure (Fig. 10A and E). These data strongly confirm that BR extract activates HaCaT cell proliferation mainly through stimulating ERK1/2 and PI3K/Akt pathways.

Nevertheless, we would like to confirm whether BR extract also induces HaCaT cell migration. Therefore, we performed Transwell cell migration assay. In contrast to our previous results, we observed no significant difference in terms of cell distribution and number of cell among all control and experimental groups (Fig. 11). Altogether, our findings suggest that BR extract has an enhancing preference towards cell proliferation rather than cell migration.

4. Discussion

In response to skin injury, restoration to normal physiology of skin tissues is regulated by a complex process called wound healing [32]. The wound healing process involves inflammation phase, epithelialization phase, proliferation phase, and remodeling phase [33,34] where each of these phases requires the harmonization of specific molecular modulators, including immune cells, cytokines, and growth factors [35]. However, some conditions such as immunocompromised, diabetes, and aging may delay normal healing rate leading to an increase risk of infection or developing complications. Therefore, discovering novel agents

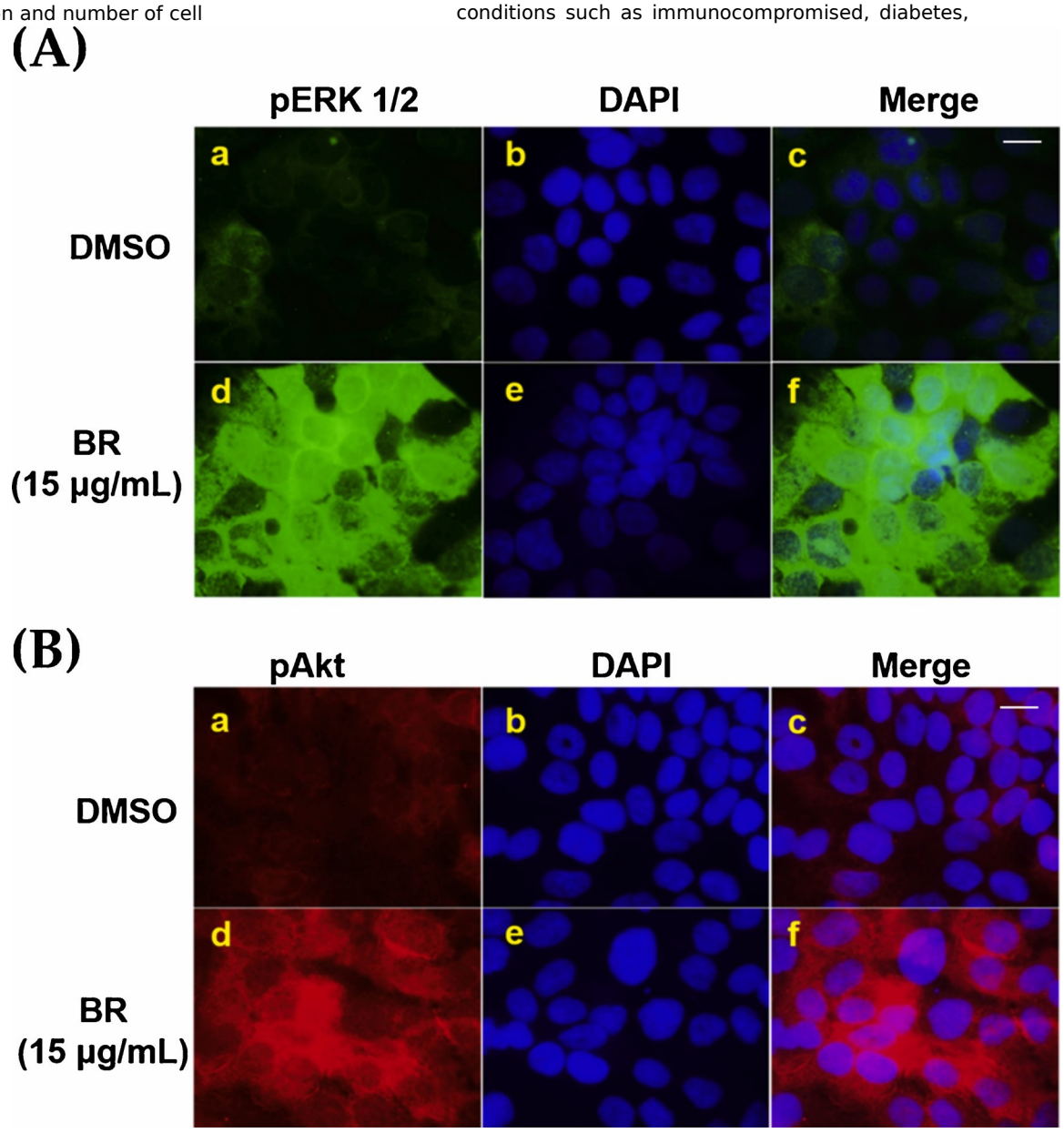


Fig. 8. Effects of BR extract on activating growth and survival signal transduction pathways in individual HaCaT cells. Immunostaining for pERK1/2 (green) (**A**) and pAkt (red) (**B**) in the cells incubated with BR extract at 15 μg/mL for 15 min. Nuclei (blue) were counterstained with DAPI. DMSO was used as a negative control. Micrographs were captured by a fluorescent microscope (100× magnification, scale bar =200 μm). The images are representative of 3 individual experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

and development of wound dressing that facilitate wound healing by stimulating intracellular healing factors would be beneficial for facilitating regeneration.

Many plants have long been used for wound healing, and the association between anti-inflammatory effects of some plants and wound healing has been reported [36]. *Boesenbergia rotunda* (BR) is one of the most popular traditional herbs in Thailand. The pharmacological and biological effects of BR extract on accelerating wound healing in animal models were examined by the excision wound model in rats. The results from these studies obviously showed that BR extract could reduce times required for wound healing, increase percentage of wound contraction, and increase period of epithelialization. Moreover, histological section showed that BR ethanolic extract treated group presented narrow scar region of wound closure [31,37]. These previous studies provide convincing data suggesting that BR possesses wound-healing promoting ability. Although these previous reports revealed promising results that BR helped heal skin wound, studies related to its potential mechanisms of action at the molecular level have not been elucidated. Therefore, our current study focused mainly on identifying specific signal transduction pathways that are activated in response to BR extract treatment. As a step towards the understanding about how BR modulates cellular behavioral changes to facilitate skin wound healing, we initiated our study by performing cell viability assay in human keratinocyte cells, HaCaT, that were treated with various concentrations of BR extract for 48 h in complete media or in serum-free media. Our results demonstrated that the extract with concentrations higher than 31.3 μg/mL significantly affected the viability of HaCaT cells. These events are likely the consequences of apoptotic cell dead induction. To support our statement, we observed that the morphology of cells under the light microscope was changed to blebbing characteristics over the course of the extract incubation (data not shown). These results suggest that BR extract at too high concentrations may create a decent degree of intracellular stress that eventually stimulates cell apoptosis. This phenomenon reflects the importance of drug or treating agent optimization for the best efficacy with the lowest toxicity. In our current study we aimed to select specific concentrations in the non-toxic ranges for all experiments to ensure that the cell viability of HaCaT cells is not interfered. Interestingly, we

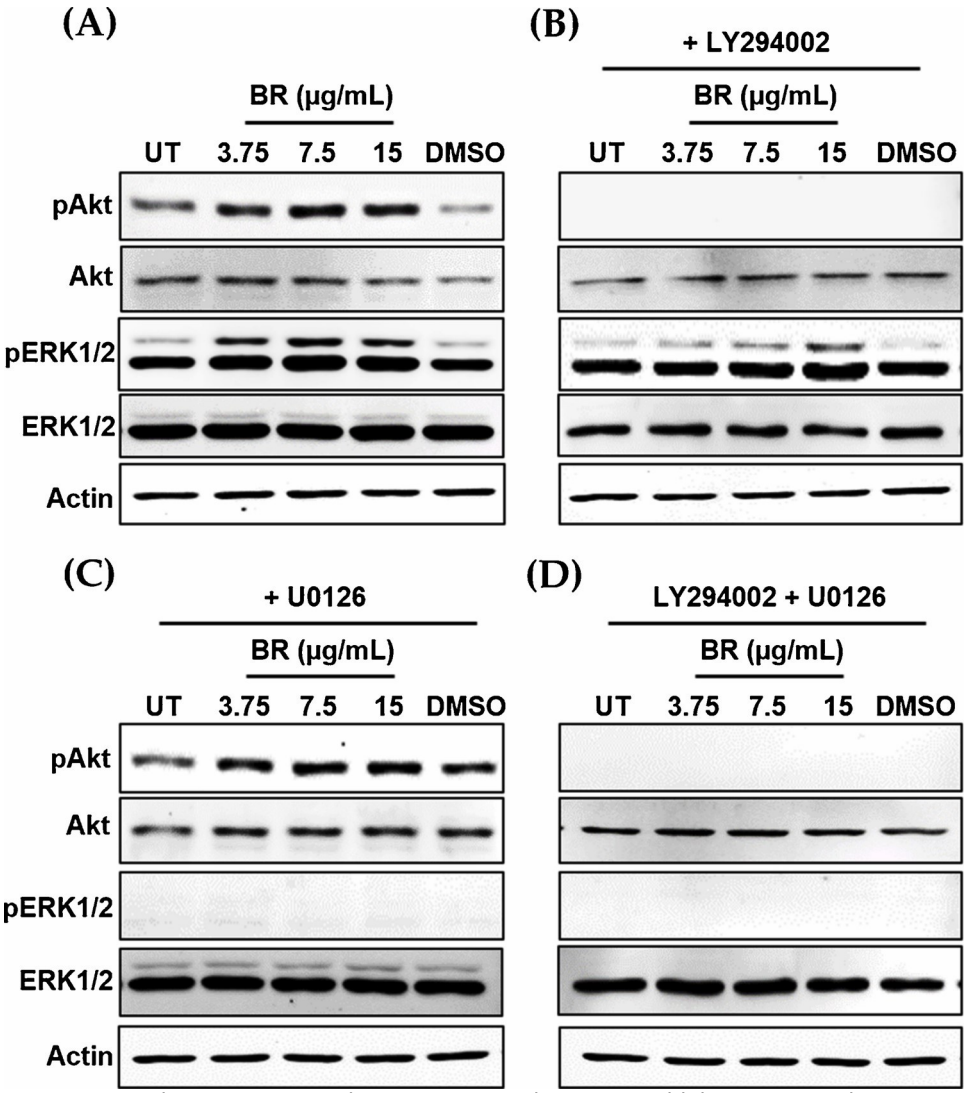


Fig. 9. Western blot analysis of pERK1/2 and pAkt upon stimulation with BR extract with or without the presence of specific inhibitors of growth and survival signaling. (**A**) Detection of pERK1/2 and pAkt after incubation with BR extract at various concentrations (3.75, 7.5, and 15 μg/mL) for 15 min. (**B**) Detection of BR extract-treated HaCaT cells with the presence of PI3K inhibitor (LY294002). (**C**) Detection of BR extract-treated HaCaT cells with the presence of MEK inhibitor (U0126). (**D**) Detection of BR extract-treated HaCaT cells with the presence of LY294002 and U0126. DMSO was used as a vehicle control, and actin was used as an internal control. Data are the representatives of 3 independent experiments.

observed that HaCaT cells cultured in serum-free media and treated with increased concentrations of BR extract (up to 15.6 μg/mL) exhibited an increase in cell viability. In contrast, the treated cells cultured in complete media showed no changes in cell viability when similar concentrations of BR extract were applied. These phenomena can be explained by the fact that complete media contain high level of several different growth factors and molecules that strongly stimulate cell growth and survival, and that may obscure the authentic effects of BR extract. When fetal bovine serum was excluded, the effects of BR extract on increasing cell viability (or metabolic activity) were clearly seen. From these surprising results, it can be interpreted that the rising in cell viability may be the result of increasing cell proliferation and survival. As expected, data from colony forming assay and cell counting verified that the events observed in MTT assay were the consequence of induction of cell proliferation since we observed a significant increase in cell-occupied areas and increased number of HaCaT cells treated with increased concentrations of BR extract. Additionally, Ki-67 protein which is a cellular marker for proliferation [38] was found to be tremendously increased in the nuclei of BR extract-treated cells. The expression of this nuclear protein is important for normal S-phase progression in human cells [39]. Therefore, our findings disclosed that BR extract can induce cell proliferation in HaCaT cells, at least in part through upregulating Ki-67 protein.

We further explored the possible mechanism of action of BR extract at the molecular level by tracking the early signal transduction pathways relevant to cell proliferation. One possible signaling pathway responsible for stimulating cell proliferation is the MAPK/ERK pathway. The activity of MAPK is regulated by the reversible phosphorylation of

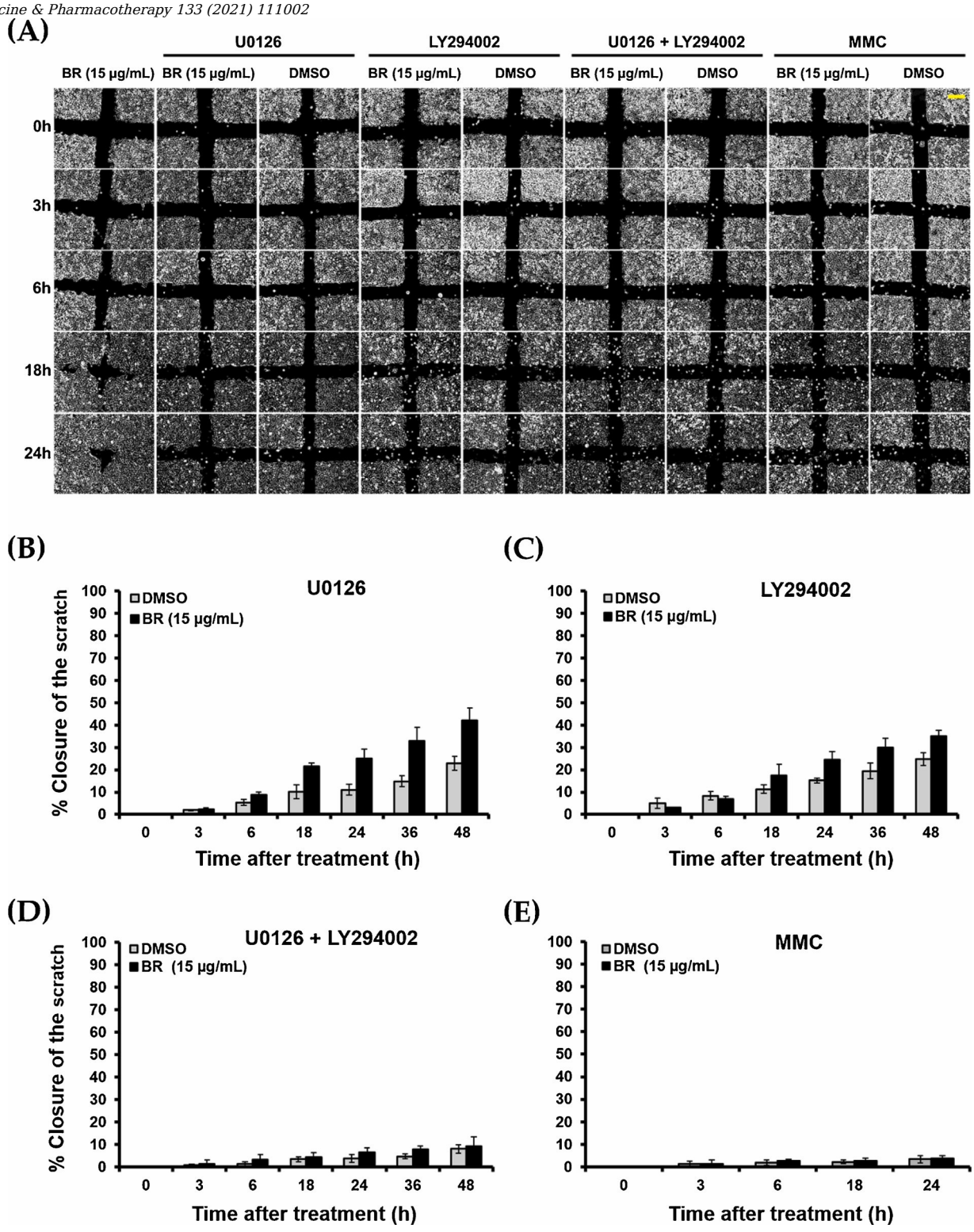


Fig. 10. Scratch wound healing assay for determining the healing-promoting effects of BR extract (15 μg/mL) in the presence of specific inhibitors of growth and survival signaling. (**A**) Phase-contrast microscopy (10× magnification, scale bar =200 μm) of scratch wound healing assay with U0126, LY294002, combination of U0126 and LY294002, and mitomycin C (MMC) over the course of 24 h. (**B**) Quantitative analysis of percent closure of the scratch-wounded areas of HaCaT cell monolayer treated with BR extract with the presence of U0126. (**C**) Quantitative analysis of percent closure of the scratch-wounded areas of HaCaT cell monolayer treated with BR extract with the presence of LY294002. (**D**) Quantitative analysis of percent closure of the scratch-wounded areas of HaCaT cell monolayer treated with BR extract with the presence of U0126 + LY294002. (**E**) Quantitative analysis of percent closure of the scratch-wounded areas of HaCaT cell monolayer treated with BR extract with the presence of MMC. DMSO was used as a negative control. Data are from 3 different experiments.

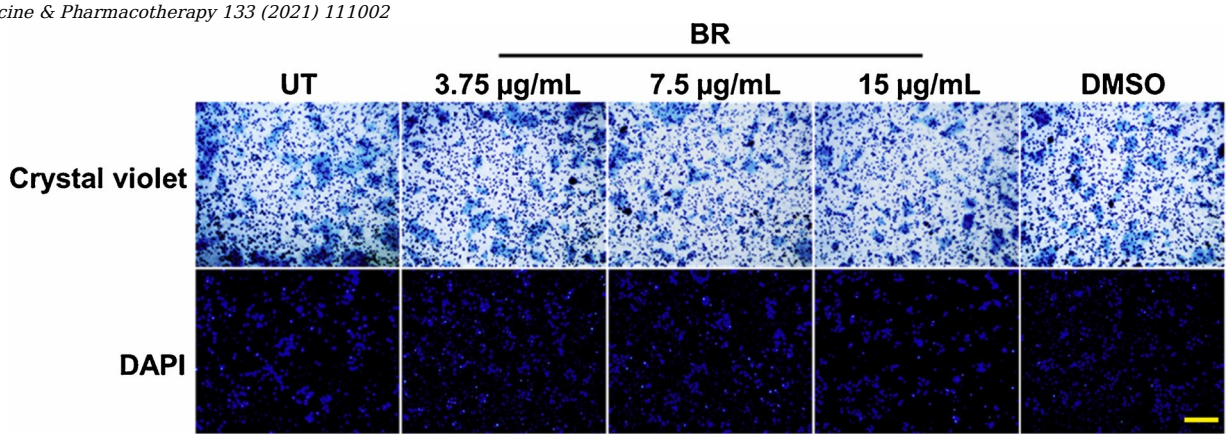


Fig. 11. Effects of BR extract on cell migration. Transwell migration assay of HaCaT cells treated with various concentrations of BR extract. The migrating cell attached on the other side of the upper chamber were stained with crystal violet and DAPI (for nuclear staining). Micrographs (10× magnification, scale bar =200 μm) were captured under a phase-contrast microscope and a fluorescent microscope for crystal violet staining and DAPI staining, respectively.

specific tyrosine and threonine residues, and active ERK accumulation in the nucleus is critical in cell cycle progression through G1 [40]. Therefore, we examined the phosphorylation status of ERK1/2 at various time points over the course of 24 h and found that BR extract rapidly activated ERK1/2 after 2 min of addition. The maximum phosphorylation was seen at 15 min post-treatment. Surprisingly, we noticed a second wave of ERK1/2 phosphorylation where the phosphorylation status was raised followed by dephosphorylation of the kinase which is likely to be the normal negative feedback mechanism of the kinase/phosphatase system. However, BR extract did not activate the phosphorylation of other MAP kinases including c-Jun N-terminal kinases (JNK) or P38 mitogen-activated protein kinases (P38). These data indicate that BR extract can specifically induce the proliferative signaling of ERK1/2 kinase in HaCaT cells. In additional to MAPK signaling cascade, we examined whether the PI3K/Akt is also induced by BR extract. The rationale behind this is that remarkable progress has been made in defining how Akt promotes normal cell cycle progression. Specifically, it is now well known that activated Akt kinase regulates the function of diverse substrates related to cell cycle progression at the G1/S and G2/M transitions, either by direct phosphorylation of the target proteins themselves or, indirectly, by regulating protein expression levels [41]. Our data clearly showed that BR extract could also induce Akt phosphorylation in a manner similar to that of ERK1/2. Additionally, data from immunofluorescence study demonstrated that the intracellular phosphorylation of ERK1/2 and Akt occurred in individual cells of HaCaT treated with BR extract. Altogether, our results strongly suggest that BR extract rapidly induces growth and survival via activating ERK1/2 and Akt early signal transduction pathways. To firmly address this claim that the MAPK and PI3K/Akt induction are associated with proliferative behavior of HaCaT treated with BR extract, we used a specific inhibitor of MEK (U0126), a specific inhibitor of PI3K/Akt (LY294002), or a combination of these two inhibitors to inhibit the relevant signaling. Data from Western blot analysis showed that each of these two inhibitor could dramatically inhibit BR extract-induced phosphorylation of its relevant signaling pathway, and the combination of U0126 and LY294002 could completely inhibit both MAPK and PI3K/Akt. Consistently, our functional test confirmed that inhibition of MAPK alone or PI3K/Akt alone intensely inhibited the closure of the scratch wound. Undoubtedly, effective suppression of these two signaling pathways by the combination of U0126 and LY294002 completely blocked the ability of BR extract in inducing scratch wound healing of the HaCaT monolayer. Furthermore, we definitely clarified the proliferative effects of BR extract by using an agent, mitomycin C (MMC), that inhibits DNA synthesis [42]. We found that MMC alone completely suppressed HaCaT cell scratch wound healing. These data strongly suggest that BR extract functions to facilitate monolayer wound healing of human keratinocytes mainly through enhancing the cell proliferation but less likely through promoting cell migration. To ensure our thought, we performed Transwell cell migration assay and confirmed that BR extract did not directly promote HaCaT cell migration. The proliferative effects of BR extract may be caused by one of its active compounds, kaempferol, which was previously revealed to exhibit strong wound healing effects in diabetic and nondiabetic rats [30]. Moreover, several different kaempferol derivatives identified to be major constituents of the leaves and twigs of *Elaeagnus umbellate* have been reported to promote HaCaT proliferation [43]. Therefore, our study suggests that BR extract, containing kaempferol, exerts its potent wound healing properties through stimulating proliferative signaling pathways. Our findings defined the molecular mechanism of action of BR and verified its potential benefits in skin regeneration and wound healing in human skin.

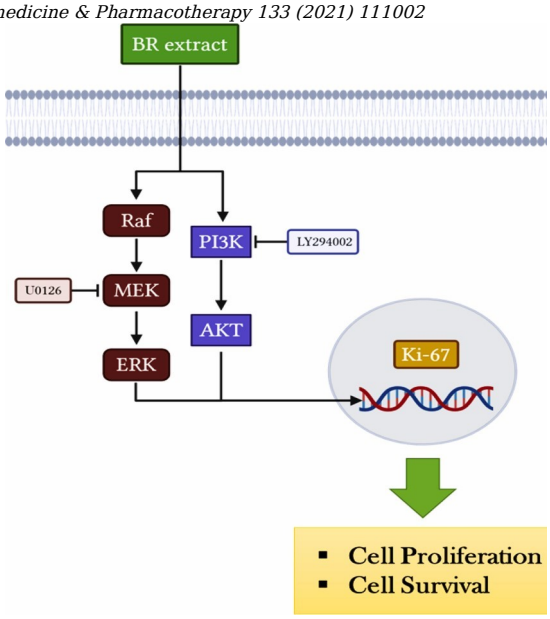


Fig. 12. Schematic figure proposing that BR extract modulates the cell proliferation and survival of HaCaT cells. BR extract drastically increases the activation of both PI3K/AKT signal transduction pathway and ERK1/2 MAPK signaling pathway, resulting in the upregulation of cell proliferation and cell survival of human keratinocytes.

5. Conclusion

We discovered that the extract from *Boesenbergia rotunda* induces the proliferation of human keratinocyte cells. Its mechanism of action, as proposed in Fig. 12, is mediated through the activation of ERK1/2 and Akt which generally function in maintaining cellular proliferation and survival. Our findings strongly support the previous reports on the wound healing property of BR extract in animal models. Overall, the current study sheds new light on the wound healing-promoting properties of BR extract at the molecular level and further supports its potential therapeutic applications for skin regeneration.

Author contributions

W.N. founded the research project, designed the experiments, did experiments, analyzed the data, wrote the manuscript, and contributed to the funding of the project. J.R. performed experiments, data analysis, and manuscript writing. N.W. analyzed the data, provided technical supports, and revised the manuscript. S.O. prepared the plant extract. P. P. and P.B. characterized the extract. M.N.T. wrote some part of the manuscript. S.P. provided some financial supports, revised the manuscript, and gave suggestions for the project. All authors have read and agreed to the published version of the manuscript.

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Declaration of Competing Interest

The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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